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We showed previously that exposure to microcystin causes eicosanoid release from rat hepatocytes. That study was extended further to test the effect of glucocorticoids on microcystin-induced release of [^{14}C] arachidonic acid and its metabolites. Treatment of hepatocyte cultures with either microcystin (1 μM) or steroids had no effect on cell viability or total cell protein. Total radioactivity released into the incubation medium was not effected by glucocorticoid alone. Release of total radioactivity increased four-fold after 2-hr of incubation with microcystin. Fluocinolone pretreatment decreased the microcystin-induced synthesis and release of prostacyclin (6-keto $F_1\alpha$) by 24 $\pm$ 2.6% ($p < 0.05$) and thromboxane B_2 (TxB_2) by 39 $\pm$ 3% ($p < 0.025$). Under these experimental conditions, the quantities of prostaglandin $F_2\alpha$ and PGE_2 released were not significantly different when control and microcystin-treated cultures were compared. The half maximal inhibition (IC_{50}) values obtained from the dose-response data for the inhibition of arachidonic acid release were comparable to normal cortisol levels in humans. Dose-response curves gave the following rank order of potency: fluocinolone > dexamethasone > hydrocortisone. These results suggest that glucocorticoid therapy might be beneficial in microcystin toxicosis.

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INHIBITION OF MICROCYSTIN-INDUCED RELEASE OF CYCLOOXYGENASE PRODUCTS FROM RAT HEPATOCYTES BY ANTI-INFLAMMATORY STEROIDS

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KEY WORDS Arachidonic acid; Steroids; Hepatocytes; Metabolism; Prostaglandin; Microcystin; Toxin

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Abstract. We showed previously that exposure to microcystin causes eicosanoid release from rat hepatocytes. That study was extended further to test the effect of glucocorticoids on microcystin-induced release of [^{14}C] arachidonic acid and its metabolites. Treatment of hepatocyte cultures with either microcystin (1 μM) or steroids had no effect on cell viability or total cell protein. Total radioactivity released into the incubation medium was not affected by glucocorticoid alone. Release of total radioactivity increased four-fold after 2-hr of incubation with microcystin. Fluocinolone pretreatment decreased the microcystin-induced synthesis and release of prostacyclin (6-keto $F_1\alpha$) by 24 ± 2.6% ($p < 0.05$) and thromboxane B_2 (TxB_2) by 39 ± 3% ($p < 0.025$). Under these experimental conditions, the quantities of prostaglandin $F_2\alpha$ and PGE_2 released were not significantly different when control and microcystin- treated cultures were compared. The half maximal inhibition (IC_{50}) values obtained from the dose-response data for the inhibition of arachidonic acid release were comparable to normal cortisol levels in humans. Dose-response curves gave the following rank order of potency: fluocinolone > dexamethasone > hydrocortisone. These results suggest that glucocorticoid therapy might be beneficial in microcystin toxicosis.

Microcystis aeruginosa algal extracts contain a known toxic cyclic peptide, microcystin, which causes death in several mammalian species. Microcystin is a hepatotoxin; however, sensitivities of non-hepatic tissues tc microcystis extracts have also been reported (1). Pronounced biochemical and morphological changes have been observed in the livers of animals exposed to microcystin; pathologic changes include congestion, edema, and inflammatory cell infiltration (2-5). The liver is involved in the pathogenesis of a number of inflammatory diseases and also represents a cellular defense system against exogenous toxins in the body (6,7). Hepatic cells have the capacity to synthesize large quantities of arachidonic acid metabolites (8-10). We have previously shown that microcystin stimulates the cyclooxygenase pathway of arachidonic acid metabolism (11).

Steroids are now used extensively in the treatment of inflammation and immune disorders. In addition, glucocorticoids have been shown to exert anti-inflammatory effects by inhibiting phospholipase A_2 activity (12-14), reducing the release of arachidonic acid metabolites, or reducing tissue prostaglandin synthesis by inhibiting prostaglandin synthetase or accelerating the breakdown of prostaglandins (15). Investigators have also suggested that impairment of prostanoid synthesis by steroids in microcystin-challenged rats reduces lethality (5,16). The mechanism of action of the toxin in hepatic tissue is not yet well understood.

In the present study, we report that treatment of rat hepatocytes with microc in promoted a significant, rapid release of prostanoids into the incubation medium, the release could be blocked by pretreatment of cells with glucocorticoids.

Materials and Methods

Hepatocyte cultures: Hepatocytes from 200-280 g, male Fisher rats (Charles River, Wilmington, MA) were isolated and cultured in Liebovitz's medium containing 18% heat-inactivated fetal calf serum (FCS) in 35 x 10 mm culture plates (17). After overnight culture at 37°C in 5% CO_2 and 95% air, only places with ≥ 90% cells attached were used. Attached cells formed a uniform monolayer and had the characteristic polygonal shape of hepatocytes.

Labeling and stimulation of hepatocytes: After 24 hr of incubation, culture medium was removed and cells were washed with Hanks balanced salt solution (HBSS). Fresh medium (1 ml) with 10% FCS, was then added along with the test steroid (Sigma chemical Co., St. Louis, MO) and 10 µmoles [^{14}C] arachidonic acid (sp act 52.7 mCi/mmole, New England Nuclear, Boston, MA). Sixteen hours later, the cells were washed and further incubated for 60 min with medium 199 containing 10% FCS, to remove unincorporated [^{14}C] arachidonic acid, then washed three times with HBSS. Finally, microcystin in HBSS containing 0.1% bovine serum albumin was added and cells were incubated for 2 hr.

Extraction of prostaglandins: Arachidonic acid and its metabolites from the incubation medium were extracted with 6 ml of chloroform:methanol (2:1,v/v) containing 0.005% butylated hydroxy toluene (Sigma Chemical Co., St.Louis, MO). Cells were then washed with 2 ml of chloroform. The chloroform phases were combined, the total lipid extract was evaporated to dryness under

nitrogen, and the samples were stored under nitrogen at -20°C. Lipid residues were dissolved in 100 µl of chloroform:methanol (2:1,v/v) and aliquotes were counted for total radioactivity release from hepatocytes. Arachidonic acid and its metabolites were resolved on thin-layer chromatography (TLC) plates precoated with silica gel-60 (E. Merck, American Scientific products, Columbia, MD). A solvent system of ethyl acetate-formic acid (80:1,v/v) was used. Spots, which migrated with authentic standards of prostaglandins (Sigma Chemical Co.,St. Louis, MO), were identified by exposure to iodine vapor, scraped, eluted with methanol, and their radioactivity quantitated by scintillation spectrometry. Protein was measured by the Lowry method (18).

Data analysis: Raw data were expressed as DPM/mg protein. Effects of glucocorticoids were determined by comparing changes in radiolabeled phospholipids, arachidonic acid, or arachidonate metabolites between control and steroid-pretreated cells after microcystin challenge. In each experiment, the average value of duplicate cultures for each treatment group was used. Statistical significance was determined by calculating the differences between control, microcystin-treated, and steroid treated groups by a paired "t" test.

RESULTS

Microcystin-induced hepatocyte arachidonic acid uptake and release: Changes in phospholipid total radioactivity and arachidonic acid release were measured after microcystin (1 μM) exposure stimulation for 2 hr. Microcystin-treated hepatocytes showed a decrease ($p < 0.005$) in total phospholipid radioactivity (116,665 ± 3,265 DPM/mg protein, n = 12) as compared to untreated cells (138,314 ± 3,327 DPM/mg protein, N = 13). In control hepatocytes, total radioactivity released represented approximately 7% of the labeled phospholipid fraction (arachidonic acid uptake); whereas in microcystin-treated cells, 27% of the total radioactivity was released (column A vs B, Table I). When free arachidonic acid was separated by TLC, approximately 50% (control) and 63% (microcystin-treated) of total released radioactivity was in the free form (column B vs C, Table I). Treatment of hepatocyte cultures with either microcystin (1 μM) or steroids had no effect on cell viability or total cell protein.

Arachidonic acid metabolism: Microcystin treatment not only reduced activity in cellular phospholipid, but also stimulated total radioactivity release by 338% and free arachidonic acid release by 430% as compared to control cultures (Table I). Prostacyclin (6-keto $F_1\alpha$) and thromboxane (TxB_2) were the major cyclooxygenase metabolites synthesized by rat hepatocytes in response to microcystin challenge. Microcystin-induced changes in release of radiolabeled arachidonic acid and its metabolites

from hepatocytes are illustrated in Fig. 1. Compared with control values, microcystin treatment (1 μM for 2 hr) stimulated the release of 6-keto $F_1\alpha$ by 19 ± 1.2% ($p < 0.05$) and TxB_2 by 52 ± 1.5% ($p < 0.001$). Under these incubation conditions, $PGF_2\alpha$ and PGE_2 release was not affected (data not shown).

Effect of glucocorticoids on arachidonic acid uptake: To determine whether glucocorticoids alter the uptake of radiolabeled arachidonic acid, we treated hepatocytes with 1 μM fluocinolone, dexamethasone, and hydrocortisone for 16 hr during incubation with radiolabeled [^{14}C] arachidonic acid. This hormone treatment did not alter cell viability and there was no significant difference in cell protein content. Mean total cellular radioactivity for control hepatocytes was 14.2 ± 0.98 x 10^4 DPM/mg protein (n = 9). Hepatocytes cultured under identical conditions but treated with various glucocorticoids (1 μM) for 16 hr showed no significant change in radiolabel uptake. Mean cellular radioactivity was 13.9 ± 1.67 x 10^4, 13.1 ± 0.96 x 10^4, and 12.7 ± 1.3 x 10^4 DPM/mg protein for fluocinolone, dexamethasone and hydrocortisone respectively.

Effect of glucocorticoids on microcystin-induced arachidonic acid metabolism: Fluocinolone pretreatment of hepatocytes produced no effect on cellular uptake of radioactivity or release of total radioactivity (Table I). Treatment of cultures with fluocinolone for 2,4,8, and 16 hr produced 3.6%, 5.6%, 20.7%, and 42.7% inhibition of radiolabeled arachidonic acid release in response to microcystin treatment (1 μM). Dexamethasone and

hydrocortisone produced similar results (data not shown). In all subsequent experiments, 16 hr preincubation with glucocorticoids was used when measuring arachidonic acid metabolism.

As shown in Fig.1, fluocinolone significantly suppressed microcystin-induced release of free arachidonic acid ($p < 0.025$). Pretreatment with steroids also inhibited synthesis and release of radiolabeled 6-keto $F_1\alpha$ and TxB_2. The degree of inhibition of TxB_2 appeared to be greater (39 ± 3%) than the degree of inhibition of 6-keto $F_1\alpha$ (24 ± 2.6%). Under these experimental conditions, the quantities of $PGF_2\alpha$ and PGE_2 release were not significantly different when control and experimental cultures were compared (data not shown). Under the same conditions, dexamethasone treatment inhibited the release of arachidonic acid from phospholipid by 38 ± 3.1% ($p < 0.005$) and hydrocortisone reduced the release by 26% ± 2.9. As would be expected, dexamethasone and hydrocortisone pretreatment, under the same conditions, also inhibited TxB_2 release by 37 ± 1.4% and 29.0 ± 2.1% ($p < 0.05$), respectively. Dexamethasone and hydrocortisone did not significantly reduce microcystin-induced release of 6-keto $F_1\alpha$ (Fig. 1).

Relative efficacy of glucocorticoids as inhibitors of microcystin-induced arachidonic acid release: A rank order potency of fluocinolone, dexamethasone, and hydrocortisone as inhibitors of microcystin-induced arachidonic acid release was determined. Cultures were pretreated with glucocorticoid concentrations for 16 hr. The inhibition of microcystin-induced

release of free arachidonic acid was found to be dependent on steroid concentrations and reached a maximum of 50% at 10^{-3}M or less. The half maximal inhibition (IC_{50}) of arachidonic acid release was calculated from Fig. 2. The values for fluocinolone, dexamethasone and hydrocortisone were 3 x 10^{-8}M, 1 x 10^{-6}M, and 9 x 10^{-6}M, respectively.

DISCUSSION

In this study we showed that microcystin, a known toxic and potentially inflammatory agent (5,12), increased the release of arachidonic acid from membrane phospholipid and induced the synthesis of its metabolites in the incubation medium. Microcystin stimulated a large increase in TxB_2 and a moderate increase in prostacyclin release from rat hepatocytes. In addition, 16-hr pretreatment with glucocorticoids significantly reduced the microcystin-induced release of these eicosanoids.

Although the mechanism for the effect of microcystin on the release of arachidonic acid metabolites is unknown, it has been suggested that inhibitors of sulfhydryl enzymes could block the re-uptake of free arachidonic acid into lipid pools, resulting in an increase of prostaglandin synthesis (19). Therefore, re-esterification of liberated arachidonic acid would be a controlling factor in the balance of free arachidonic acid and production of its metabolites. We reasoned that microcystin may alter the sulfhydryl enzymes active in phospholipid reacylation. In this study, about 63% of radioactivity liberated by

microcystin was detected as free arachidonic acid and was not re-incorporated into membrane phospholipid. Microcystin at a very low concentration (10 nM) stimulated the cells to release 71% of free arachidonic acid, with no change in metabolite synthesis and release (unpublished data), suggesting, at least a partial block of re-esterification of arachidonic acid, probably mediated via inhibition of sulfhydryl arachidonyl Co-enzyme A synthetase and acyl transferase activities.

Steroids are widely used as anti-inflammatory agents, due in part to their ability to inhibit the hydrolytic release of arachidonic acid from phospholipids by inducing a phospholipase A_2 inhibitory protein termed lipomodulin (13,15). Previously, Shohami and others (20) have reported that dexamethasone provides protective effects in T-2 toxicosis; this was probably mediated via suppression of prostaglandin synthesis and release. The molecular basis for the protective effects of steroids in prevention of pathologic change remains obscure.

The present study was initiated to determine the effects of glucocorticoids on the uptake and release of radiolabeled arachidonic acid by rat hepatocytes in response to microcystin exposure. Data show that microcystin stimulated the synthesis and release of arachidonic acid and its metabolites. The effect was partially blocked by pretreatment of cultures with glucocorticoids. Pretreatment of hepatocytes with glucocorticoids resulted in a moderate decrease in the uptake of radiolabeled arachidonic acid by the phospholipid pool. Steroids also

inhibited the release of this unsaturated fatty acid when hepatocytes were treated with microcystin. The long duration of pretreatment essential for the inhibition of arachidonic acid release, the persistence of the effects after steroid removal from the incubation medium during toxin exposure, and the specificity of the response to different steroids suggest that the effect may be dependent on a receptor-mediated process involving new protein synthesis (21). The degree of inhibition of TxB_2 synthesis (42.8 ± 6.1%) compared to the inhibition of arachidonic acid release from cellular phospholipid (37.4 ± 3.9%) suggests that fluocinolone blocks both PLA_2 and thromboxane synthetase. Similar findings have been reported earlier in rat macrophages (22,23). These authors suggested the possibility that glucocorticoids may exert their effects, not only on phospholipase A_2 levels, but also at steps regulating prostaglandin synthetic and/or degradative enzymes. This hypothesis has not been tested in hepatocytes.

Dose-response data in Fig. 2 illustrate a rank order of potency for the three steroids tested. Fluocinolone was the most potent in inhibiting arachidonic acid release induced by microcystin, followed by dexamethasone and hydrocortisone. These findings are similar to what has been observed for a variety of biological effects of these hormones (24,25). Furthermore, the IC_{50} values calculated from the dose-response data for the inhibition of arachidonic acid release are comparable to normal cortisol levels in humans (hydrocortisone 10^{-7}M). This in-vitro

determination of glucocorticoid supression of arachidonic acid release from hepatocytes may be of value in predicting in-vivo anti-inflammatory efficacy. The clinical relevance of toxin-specific inhibition of arachidonic acid release by glucocorticoid-treated hepatocytes remains to be established, but evaluation of steroid treatment of specific-toxin-exposed clinical populations should be considered. The data presented here suggest that microcystin-induced inflammation may involve regulation of arachidonic acid metabolism in hepatocytes. Such changes may be important in the pathogenesis of toxin-induced injury.

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Table I. Effect of Microcystin and Fluocinolone on Uptake and Release of Radioactivity by Hepatocytes

	Cellular Radioactivity (A)	Total Released Radioactivity (B)	[^{14}C] Released Arachidonic Acid (C)
Control (13)	138,314 ± 3,307	9,273 ± 1,050	4,573 ± 761
Fluocinolone (9)	137,085 ± 2,630	8,719 ± 1,032	5,641 ± 528
Microcystin (12)	116,665 ± 3,265 (<0.005)	31,415 ± 6,307 (<0.001)	19,688 ± 1,468 (<0.005)
Microcystin+ Fluocinolone (6)	124,990 ± 4,718 (<0.001)	24,839 ± 2,342 (<0.001)	13,629 ± 874[a] (<0.05)

Pretreatment of hepatocytes with 1 μM fluocinolone for 16 hr. Prelabeled cells were exposed to 1 μM microcystin for 2 hr. Cellular radioactivity (arachidonic acid uptake) associated with phopholipid was decermined by washing monolayers with HBSS to remove radioactivity associated with cell surface. Cells were digested with 1N NaOH and an aliquote counted for cellular radioactivity (Coluan A). Medium was collected and counted (column B); medium was extracted and arachidonic acid release was determined by TLC (column C). The data represent DPM/mg protein (mean ± SEM) of the number of experiments in parenthesis. P-value in parenthesis.

[a] p-significant at least < 0.025 from microcystin-treated cultures alone.

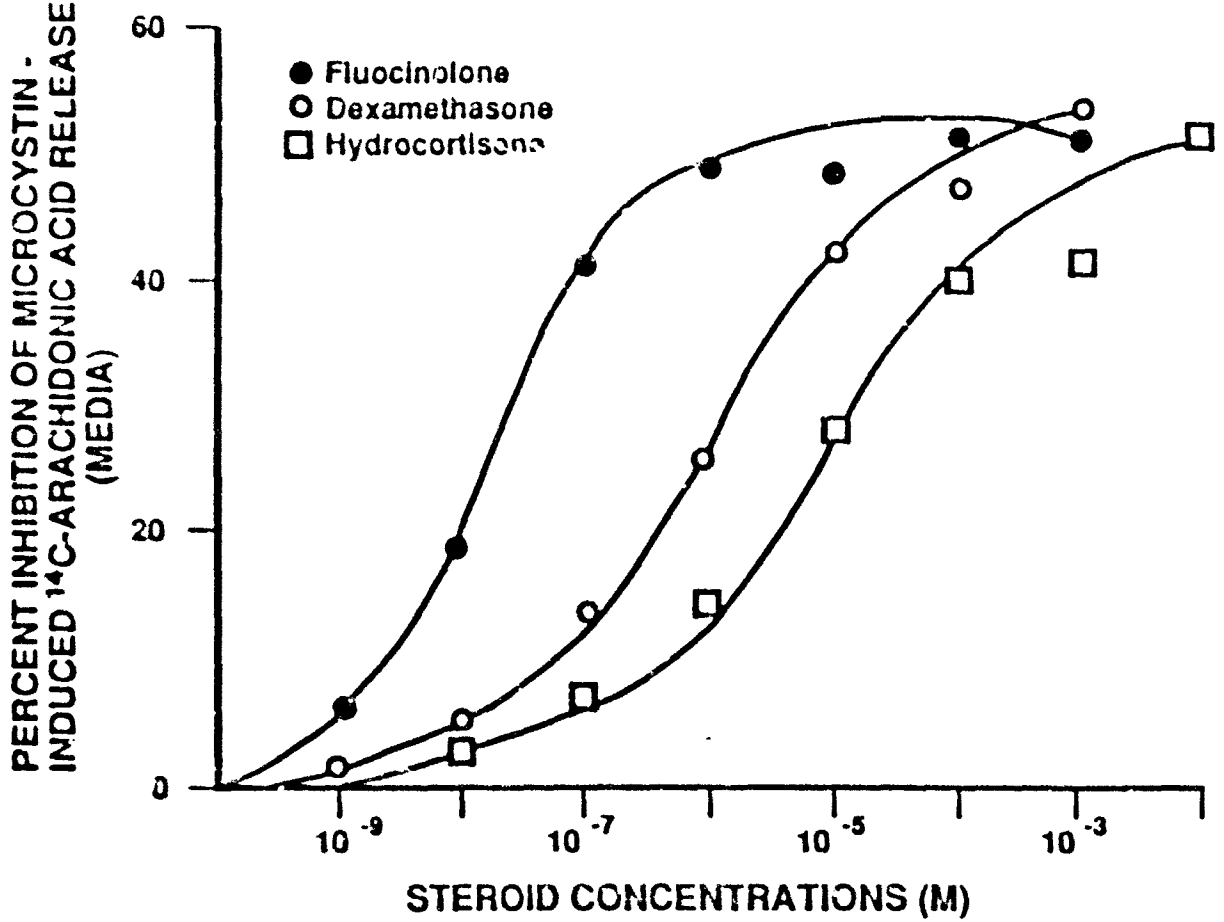
Legend

Figure 1. Effect of fluocinolone, dexamethasone, and hydrocortisone on hepatocyte arachidonic acid release and metabolism. Microcystin challenge (1 μM for 2 hr) resulted in radiolabeled arachidonic acid release and radiolabeled metabolite production. These parameters were inhibited by pretreatment for 16 hr with 1 μM steroids. Data points represent mean values from duplicate determinations of three to six separate experiments. Microcystin-induced changes in radioactivity were compared for fluocinolone, dexamethasone, and hydrocortisone-treated and untreated cells by means of a paired "t" test.

(*) - p at least <0.05 from control.

(+) - p at least < 0.05 from microcystin-treated cultures.

Figure 2. Dose-response relationships for glucocorticoid inhibition of microcystin-induced arachidonic acid release. The glucocorticoids pretreatment period was 16 hr. Data points represent mean values from duplicate determinations in three to five separate experiments. Standard deviation in these experiments was always within 10% of the mean.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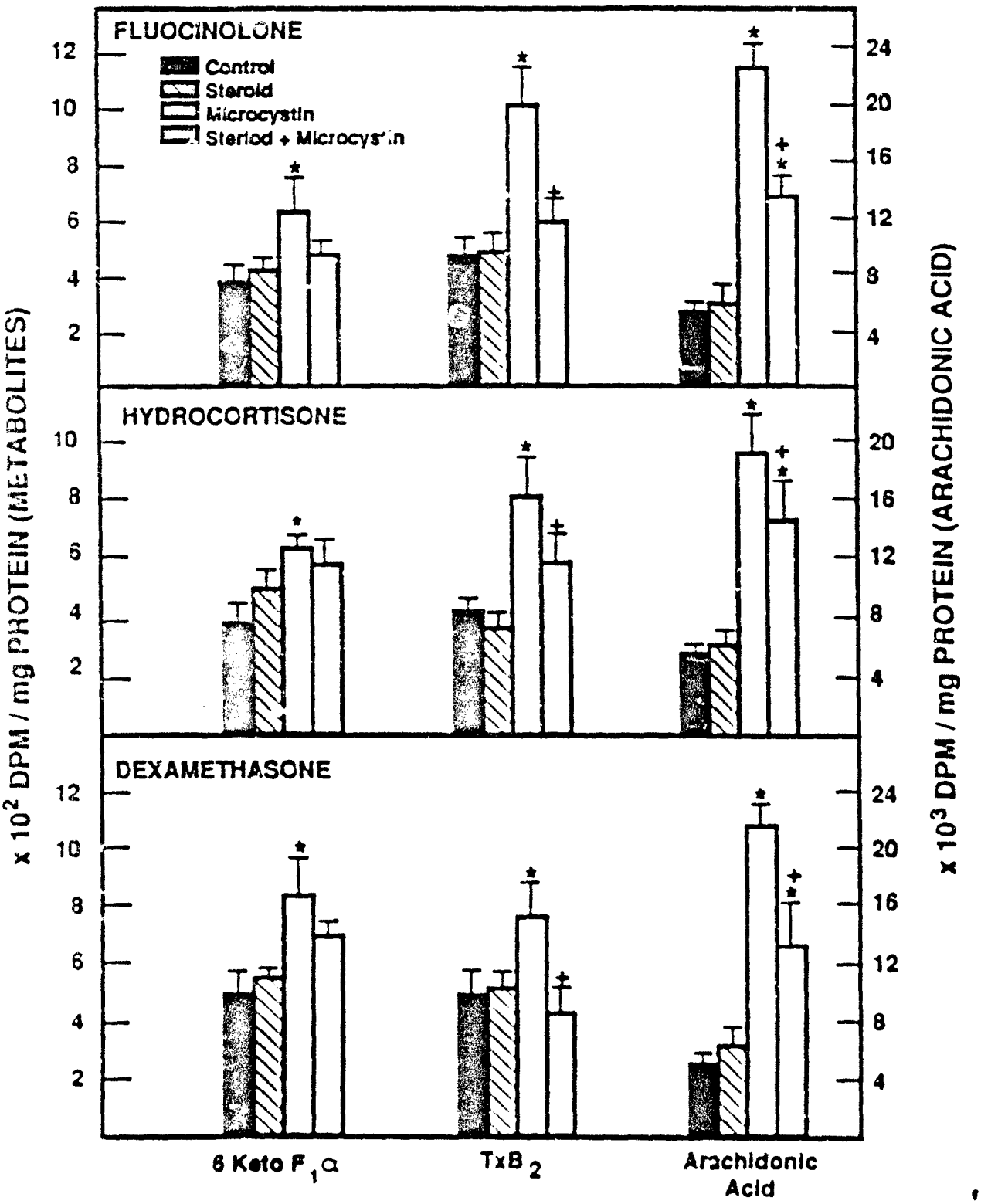
FLUOCINOLONE
Control
Steroid
Microcystin
Steriod + Microcystin
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